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(54) CEA derivatives lacking the Hydrophobic C-terminal domain.

(57) The present invention provides recombinant CEA glycoprotein derivatives and to methods for their production. These recombinant CEA glycoprotein derivatives are characterized in that they are free from cross-reactive CEA-like antigens, antigenically indistinguishable from the soluble form of CEA shed from tumor cells and devoid of ethanolamine. Said derivatives preferably have the amino acid sequence [SEQ ID NO: 1]. The said CEA glycoprotein derivatives may be used as reagents in an immunoassay for the diagnosis of neoplastic diseases. The invention also relates to a DNA encoding a said recombinant CEA glycoprotein derivative such as the DNA having the nucleotide sequence [SEQ ID NO: 2] or a functional equivalent sequence thereof. The present invention also relates to recombinant vectors comprising a said DNA, which recombinant vector is capable of directing the expression of the said DNA in a compatible host cell and to transformed host cells containing such a recombinant vector. The recombinant CEA glycoprotein derivative of the present invention, preferably integrated in a test-kit may be used for determining the presence of tumor cells in a sample of body fluid.

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The present invention relates to derivatives of the carcinoembryonic antigen (CEA).

Carcinoembryonic antigen (CEA) is one of the most studied human tumor markers and is widely used in the diagnosis of neoplastic diseases, such as colorectal cancer. Thus, e.g. when the serum levels of CEA are elevated in a patient, a drop of CEA levels after surgery means that the resection of the tumor was successful. On the other hand a subsequent rise in serum CEA levels after surgery indicates that metastases of the original tumor may have formed or that new primary tumors have grown. For a review see Shively J. E. and Beatty J. D., "CEA-related antigens: molecular biology and clinical significance", *Crit. Rev. Oncol. Hematol.* 2, 355-399 [1985]; and Mach J. -P., Pèlegrin A. and Buchegger F., "Imaging and therapy with monoclonal antibodies in non-hematopoietic tumors", *Curr. Opin. Immunol.* 3, 685-693 [1991].

10 The complete cDNA sequence encoding the CEA protein codes for a polypeptide of 702 amino acids consisting of a 34 amino acid residues long leader peptide, a 108 amino acid residues long NH₂-terminal domain, three homologous repeating domains of 178 amino acid residues and a hydrophobic C-terminal domain of 26 amino acid residues (Zimmermann W., Ortlieb B., Friedrich R. and von Kleist S., "Isolation and characterization of cdna clones encoding the human carcinoembryonic antigen reveal a highly conserved repeating structure", *Proc. Natl. Acad. Sci. U S A* 84, 2960-2964 [1987]; Beauchemin N., Benchimol S., Cournoyer D., Fuks A. and Stanners C. P. "Isolation and characterization of full-length functional cDNA clones for human carcinoembryonic antigen", *Mol. Cell. Biol.* 7, 3221-3230 [1987]; Oikawa S., Kosaki G. and Nakazato H., "Molecular cloning of a gene for a member of carcinoembryonic antigen (CEA) gene family: signal peptide and N-terminal domain sequences of nonspecific crossreacting antigen (NCA)", *Biochem. Biophys. Res. Commun.* 146, 464-469 [1987]). The 34 amino acid residues long leader peptide is cleaved from the precursor CEA polypeptide in the process of the transfer through the endoplasmatic reticulum membrane.

15 The hydrophobic C-terminal domain is also missing in the mature membrane-bound CEA glycoprotein. It has been shown that CEA is attached to the membrane through a phosphatidylinositol-glycan tail (PI-G) covalently linked through ethanolamine to the COOH-terminal residue of mature CEA (Hefta S. A., Hefta L. J., Lee T. D., Paxton R. J. and Shively J. E., "Carcinoembryonic antigen is anchored to membranes by covalent attachment to a glycosylphosphatidylinositol moiety: identification of the ethanolamine linkage site", *Proc. Natl. Acad. Sci. U S. A.* 85, 4648-4652 [1988]). It is generally assumed that CEA is processed post-translationally to remove the hydrophobic C-terminal domain with subsequent addition of a PI-G anchor 20 to the last amino acid of the third repeated domain. The PI-G tail can be cleaved by phosphatidylinositol-specific phospholipase C releasing the membrane-bound form of CEA. The soluble form of CEA formed in this way always comprises the ethanolamine residue coupled to the carboxy-terminus of the last amino acid sequence present in the mature form of CEA (i.e. the carboxy-terminus of the last amino acid of the third repeated domain) and possibly some fragment of the phosphatidylinositol-glycan tail.

25 30 35 It has been suggested (Caras I. W., Weddell G. N., Davitz M. A., Nussenzeig V. and Martin D. W., Jr., "Signal for attachment of a phospholipid membrane anchor in decay accelerating factor", *Science* 238, 1280-1283 [1987]; Hefta L. J., Schrewe H., Thompson J. A., Oikawa S., Nakazato H. and Shively J. E., "Expression of complementary DNA and genomic clones for carcinoembryonic antigen and nonspecific cross-reacting antigen in Chinese hamster ovary and mouse fibroblast cells and characterization of the membrane-expressed products", *Cancer Res.* 50, 2397-2403 [1990]; Hemperly J. J., Edelman G. M. and Cunningham B. A. "cDNA clones of the neural cell adhesion molecule (N-CAM) lacking a membrane-spanning region consistent with evidence for membrane attachment via a phosphatidylinositol intermediate", *Proc. Natl. Acad. Sci. U S A* 83, 9822-9826 [1986]) that the COOH-terminal domain of PI-G anchored proteins is important for their correct targeting and attachment to the cell surface. Complete or partial deletion of the hydrophobic domain can result in the secretion of mutant proteins into the medium (Udenfriend S., Micanovic R. and Kodukula K., "Structural requirements of a nascent protein for processing to a PI-G anchored form: studies in intact cells and cell-free systems", *Cell Biol. Int. Rep.* 15, 739-759 [1991]).

40 45 50 55 The standard CEA reference presently used is generally isolated from extracts of human tumors. This CEA appears to be shed from the cell surface of tumors by cleavage of the PI-G anchor (Kuroki M., Murakami M., Wakisaka M., Ikeda S., Oikawa S., Oshima T., Nakazato H., Kosaki G. and Matsuoka Y., "Immunoreactivity of recombinant carcinoembryonic antigen proteins expressed in Escherichia coli", *Immunol. Invest.* 21, 241-257 [1992]). The disadvantage of the CEA isolated from human tumor extracts is that it may contain cross-reactive CEA-like antigens which may interfere with an immunoassay of the CEA released by tumors. It is known that these CEA-like antigens are elevated in many non-cancerous conditions, such as e.g. in inflammatory liver diseases and in smokers. Efforts to overcome the problem caused by interfering CEA-like antigens lead to the cloning of the DNA encoding CEA. In EP-A-263,933 various nucleic acid sequences coding for CEA peptid sequences are disclosed.

Efforts to express different domains of the CEA molecule in bacteria showed that the CEA domains expressed in bacteria had a lower antigenicity, presumably due to incomplete folding (Kuroki M., Murakami M., Wakisaka M., Krop Watorek A., Oikawa S., Nakazato H., Kosaki G. and Matsuoka Y., "Epitopes predominantly retained on the carcinoembryonic antigen molecules in plasma of patients with malignant tumors but not on those in plasma of normal individuals", *Jpn. J. Cancer Res.* 83, 505-514 [1992]).

As an alternative to the use of human tumor extracts, it has been proposed to purify CEA from the culture medium from human cancer cell lines. It has been found however, that the complete mature form of CEA is not actively secreted, but only shed in low amounts (see below) in the culture medium of colon cancer carcinoma cell lines. One solution to overcome the problem of the low-level expression was to 10 prepare fragments of CEA. However, quite obviously the fragments often do not comprise all important epitopes which are present in CEA, i.e. the epitopes generally known as GOLD 1 - 5 described by Hammarstrom et al. (Hammarstrom S., Shively J. E., Paxton R. J., Beatty B. G., Larson A., Ghosh R., Bormer O., Buchegger F., Mach J. -P., Burtin P., Seguin P., Darbouret B., Degorce F., Sertour J., Jolu J. -P., Fuks A., Kalthoff H., Schmiegel W., Arndt R., Kloppel G., von Kleist S., Grunert F., Schwarz K., 15 Matsuoka Y., Kuroki M., Wagener C., Weber T., Yachi A., Imai K., Hishikawa N. and Tsujisaki M. "Antigenic sites in carcinoembryonic antigen", *Cancer Res.* 49, 4852-4858 [1989]).

The problem to be solved by the present invention was therefore to provide a CEA derivative which is free from cross-reactive CEA-like antigens, is antigenically indistinguishable from the soluble form of CEA shed from tumor cells, i.e. comprises all important epitopes of the CEA protein and on the other hand is 20 secreted in high amounts by a recombinant host.

It has now been found that a recombinant cDNA encoding CEA which lacks the 3' region encoding the 26 amino acid hydrophobic domain is capable of secreting a CEA derivative which fulfills the requirements outlined above. Upon transfection of the said cDNA into a suitable host cell, such as a rat or a human carcinoma cell, a 50- to 100-fold higher level of secretion of a fully immunogenic CEA glycoprotein into the 25 culture medium is obtained.

Therefore, the present invention relates to a recombinant CEA glycoprotein derivative which is characterized in that it is:

- (a) free from cross-reactive CEA-like antigens;
- (b) antigenically indistinguishable from the soluble form of CEA shed from tumor cells; and
- 30 (c) secreted in high amounts in devoid of ethanolamine.

Said recombinant CEA glycoprotein derivative is secreted in high amounts in culture medium from cells transfected with said recombinant cDNA.

The term "free from cross-reactive CEA-like antigens" relates to the fact that the recombinant CEA glycoprotein derivative of the present invention is secreted by host cells transformed with a recombinant 35 vector comprising a cDNA encoding said recombinant CEA glycoprotein derivative. Because it is not isolated from tumor extracts it does not contain the cross-reactive CEA-like antigens usually present in such extracts.

The term "antigenically indistinguishable from the soluble form of CEA shed from tumor cells" means that the recombinant CEA glycoprotein derivative is immunologically the same as the natural form of CEA, 40 i.e. comprises all major epitopes present in CEA, especially that it comprises all five epitopes generally known as the GOLD 1 - 5 epitopes (Hammarstrom et al. [1989], supra).

The term "being devoid of ethanolamine" relates to the fact that the recombinant CEA glycoprotein of the present invention lacks the ethanolamine residue and possibly some fragment of the phosphatidylinositol-glycan tail normally present in the soluble form of CEA as it is obtainable from 45 untransfected tumor cells, e.g. after shedding from tumor cells or after treatment with a phosphatidylinositol-specific phospholipase. Because the cDNA encoding the recombinant CEA glycoprotein of the present invention lacks the sequence coding for the hydrophobic C-terminal tail present in the precursor form of CEA polypeptide, the polypeptide expressed from this cDNA cannot be anchored in the cell membrane. It was found that quite surprisingly the lack of the hydrophobic domain in the CEA glycoprotein does not 50 affect the transport of CEA towards the cell surface in either human or rat carcinoma cells, but only prevents its anchoring to the cell surface.

The preferred recombinant CEA glycoprotein derivative of the present invention has the following amino acid sequence:

KLTIESTPFN VAEGKEVLLL VHNLPQHLFG YSWYKGERVD GNRQIIGYVI
 5 GTQQATPGPA YSGREIIYPN ASLLIQNIIQ NDTGFYTLHV IKSDLVNEEA
 TGQFRVYPEL PKPSISSLNS KPVEDKDAVA FTCEPETQDA TYLWWVNNQS
 10 LPVSPRLQLS NGNRTLTLFN VTRNDTASYK CETQNPVSAR RSDSVILNVL
 YGPDAPTISP LNTSYRSGEN LNLSCHAASN PPAQYSWFVN GTFQQSTQEL
 15 FIPNITVNNS GSYTCQAHNS DTGLNRTTVT TITVYAEPPK PFITSNNNSNP
 VEDEDAAVALT CEPEIQNNTTY LWWVNNQSLP VSPRLQLSND NRTLTLLSVT
 20 RNDVGPYECG IQNELSVDHS DPVILNVLYG PDDPTISPSY TYYRPGVNLS
 LSCHAASNPP AQYSWLIDGN IQQHTQELFI SNITEKNGL YTCQANNAS
 25 GHSRTTVKTI TVSAELPKPS ISSNNSKPVE DKDAVAFTCE PEAQNTTYLW
 WVNGQSLPVS PRLQLSNGR TLTLFNVTRN DARAYVCGIQ NSVSANRSDP
 30 VTLDVLYGPD TPIISPPDSS YLSGANLNLS CHSASNPSPQ YSWRINGIPQ
 QHTQVLFIAK ITPNNNGTYA CFVSNLATGR NNSIVKSITV SA [SEQ ID NO: 1]
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The present invention also relates to functional equivalent recombinant CEA glycoprotein derivatives having an amino acid sequence which is related to the above amino acid sequence by deletions, insertions or substitutions without essentially changing the biological and immunological properties of the said CEA glycoprotein.

Examples of amino acid substitutions which do not substantially alter the biological and immunological properties of a protein have been described, e.g., by Neurath et al., in "The Proteins", Academic Press, New York (1979), in particular in Fig. 6 at page 14 thereof. The most frequently observed amino acid substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, 40 Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, and vice versa.

The recombinant CEA glycoprotein of the present invention may be labeled by one of the labels known in the art, e.g. by a dye, a radioactive, an enzymatic, a fluorescent or a chemiluminescent label. Preferred label is radioactively labeled iodine (^{125}I).

The recombinant CEA glycoprotein of the present invention may be used as a standard in an immunoassay for detecting CEA in a biological sample, e.g. a sample of a body fluid. The person skilled in the art is in a position to configure such an immunoassay based on the general knowledge in the field of immuno diagnostics. The use of an enzyme linked immunoassay (ELISA) is preferred. Examples for such immunoassays are described e.g. in EP-A-346,710. Enzymes used to label the recombinant CEA glycoprotein derivative of the present invention include, among others alkaline phosphatase, β -galactosidase, horseradish peroxidase, glucose-6-phosphat dehydrogenase, 3-phosphoglycerate kinase (PGK).

The present invention also relates to immunoassays for the diagnosis of neoplastic diseases in which immunoassays a recombinant CEA glycoprotein derivative of the present invention is used, preferably as a standard in form of a reagent, wherein said recombinant CEA glycoprotein derivative is mixed with an inert

carrier material. Examples for such inert carrier materials are distilled water, buffers, possibly containing stabilizers and other additives generally used in reagents for diagnostic purposes.

The present invention also relates to methods for the preparation of such reagents and to the reagents per se, as well as to test-kits for the determination of the presence of cancer cells in a biological sample.

5 5 Such a test-kit comprises in a container a recombinant CEA glycoprotein derivative in accordance with the present invention, if necessary in combination with an inert carrier material and, if necessary, additional reagents such as e.g. monoclonal or polyclonal CEA antibodies.

The recombinant CEA glycoprotein of the present invention is preferably encoded by a cDNA fragment which lacks the region coding for the C-terminal hydrophobic tail. One way to obtain such a cDNA fragment 10 is to use suitable restriction endonucleases to cut a cDNA encoding the complete form of CEA directly upstream from the region coding for the hydrophobic tail that lacks in the recombinant CEA glycoprotein of the present invention. The 3' end of the cDNA fragment is then restored by using a synthetic oligonucleotide duplex which encodes the extra nucleotides which were inadvertently cleaved off by the restriction endonuclease. Preferably said synthetic oligonucleotide duplex also comprises a stop codon, 15 which causes that the translation is terminated after the last amino acid residue in the amino acid sequence [SEQ ID NO: 1]. The restriction endonuclease Eael is the most suitable enzyme for preparing the said cDNA fragment. Unfortunately this restriction endonuclease cleaves the cDNA encoding CEA at more than one position. In order to overcome this problem a 800 base pair fragment comprising the 3' end of the CEA cDNA can be isolated by digestion with Bsu36I and XbaI endonucleases, and then cutting the 20 fragment obtained in this way with Eael separately.

Thus, the present invention provides also a DNA encoding a CEA glycoprotein derivative of the present invention, such as the DNA having the nucleotide sequence

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ATGGAGTCTC CCTCGGCCCC TCCCCACAGA TGGTGCATCC CCTGGCAGAG
5 GCTCCTGCTC ACAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCAC TG
CCAAGCTCAC TATTGAATCC ACGCCGTTCA ATGTCGCAGA GGGGAAGGAG
10 GTGCTTCTAC TTGTCCACAA TCTGCCAG CATCTTTG GCTACAGCTG
GTACAAAGGT GAAAGAGTGG ATGGCAACCG TCAAATTATA GGATATGTAA
15 TAGGAACTCA ACAAGCTACC CCAGGGCCCG CATACTGG TCGAGAGATA
ATATAACCCA ATGCATCCCT GCTGATCCAG AACATCATCC AGAATGACAC
20 AGGATTCTAC ACCCTACACG TCATAAAAGTC AGATCTTGTG AATGAAGAAG
CAAATGGCCA GTTCCGGGTA TACCCGGAGC TGCCCAAGCC CTCCATCTCC
25 AGCAACAACT CCAAACCGT GGAGGACAAG GATGCTGTGG CCTTCACCTG
TGAACCTGAG ACTCAGGACG CAACCTACCT GTGGTGGGTA AACAAATCAGA
30 GCCTCCCGT CAGTCCCAGG CTGCAGCTGT CCAATGGCAA CAGGACCCCTC
ACTCTATTCA ATGTCACAAG AAATGACACA GCAAGCTACA AATGTGAAAC
35 CCAGAACCCA GTGAGTGCCA GGCGCAGTGA TTCAGTCATC CTGAATGTCC
TCTATGGCCC GGATGCCCTT ACCATTTCCC CTCTAAACAC ATCTTACAGA
40 TCAGGGAAA ATCTGAACCT CTCCTGCCAC GCAGCCTCTA ACCCACCTGC
ACAGTACTCT TGGTTGTCA ATGGGACTTT CCAGCAATCC ACCCAAGAGC
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TCTTTATCCC CAACATCACT GTGAATAATA GTGGATCCTA TACGTGCCAA
 5
 GCCCATAACT CAGACACTGG CCTCAATAGG ACCACAGTCA CGACGATCAC
 AGTCTATGCA GAGCCACCCA AACCCCTTCAT CACCAGCAAC AACTCCAACC
 10
 CCGTGGAGGA TGAGGATGCT GTAGCCTAA CCTGTGAACC TGAGATTCA
 AACACAACCT ACCTGTGGTG GGTAAATAAT CAGAGCCTCC CGGTCACTGC
 15
 CAGGCTGCAG CTGTCCAATG ACAACAGGAC CCTCACTCTA CTCAGTGTCA
 CAAGGAATGA TGTAGGACCC TATGAGTGTG GAATCCAGAA CGAATTAAGT
 20
 GTTGACCACA GCGACCCAGT CATCCTGAAT GTCCTCTATG GCCCAGACGA
 CCCCACCATT TCCCCCTCAT ACACCTATTAA CCGTCCAGGG GTGAACCTCA
 GCCTCTCCTG CCATGCAGCC TCTAACCCAC CTGCACAGTA TTCTTGGCTG
 25
 ATTGATGGGA ACATCCAGCA ACACACACAA GAGCTTTA TCTCCAACAT
 CACTGAGAAG AACAGCGGAC TCTATACCTG CCAGGCAAT AACTCAGCCA
 GTGGCCACAG CAGGACTACA GTCAAGACAA TCACAGTCTC TGCGGAGCTG
 30
 CCCAAGCCCT CCATCTCCAG CAACAACTCC AAACCCGTGG AGGACAAGGA
 TGCTGTGGCC TTCACCTGTG AACCTGAGGC TCAGAACACA ACCTACCTGT
 35
 GGTGGGTAAA TGGTCAGAGC CTCCCAGTCA GTCCCAGGCT GCAGCTGTCC
 AATGGCAACA GGACCCTCAC TCTATTCAAT GTCACAAGAA ATGACGCAAG
 40
 AGCCTATGTA TGTGGAATCC AGAACTCAGT GAGTGCAAAC CGCAGTGACC
 CAGTCACCCCT GGATGTCTC TATGGGCCGG ACACCCCCAT CATTCCCCC
 CCAGACTCGT CTTACCTTTC GGGAGCGAAC CTCAACCTCT CCTGCCACTC
 45
 GGCTCTAAC CCATCCCCGC AGTATTCTTG GCGTATCAAT GGGATAACCGC
 AGCAACACAC ACAAGTTCTC TTTATCGCCA AAATCAGGCC AAATAATAAC
 50
 GGGACCTATG CCTGTTTGT CTCTAACTTG GCTACTGGCC GCAATAATTG

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CATAGTCAAG AGCATCACAG TCTCTGCATA G

[SEQ ID NO: 2].

It is understood that the above DNA sequence includes the codons coding for the 34 amino acid residue long signal peptide which is also present in the natural form of the gene encoding CEA, which signal peptide sequence is cleaved off during the maturation of the CEA protein. On the other hand, quite obviously, said DNA sequence does not comprise the codons encoding the C-terminal hydrophobic domain present in the natural form of the gene encoding CEA.

As indicated above a DNA having the sequence [SEQ ID NO: 2] can be prepared by using the methods of recombinant DNA technology from a cDNA coding for the full-length form of CEA. Such a DNA sequence can also be prepared by chemical synthesis and/or by combining appropriate DNA fragments in such a way that a complete DNA having the sequence of [SEQ ID NO: 2] is obtained.

Because of the degeneracy of the genetic code, it will be understood that there are many potential nucleotide sequences (functional equivalents) that could code for the recombinant CEA glycoprotein derivative having the amino acid sequence [SEQ ID NO: 1]. Therefore, the present invention also relates to a functional equivalent sequence of [SEQ ID NO: 2], which nucleotide sequence encodes a functional equivalent CEA glycoprotein derivative as referred to above. Such a functional equivalent nucleotide sequence may readily be prepared using appropriate synthetic oligonucleotides in primer-directed site-specific mutagenesis on the exemplary cDNA of this invention [SEQ ID NO: 2], as described by Morinaga Y., Franceschini T., Inouye S. and Inouye M., "Improvement of oligonucleotide-directed site-specific mutagenesis using double-stranded plasmid DNA", *Bio/Technology* 2, 636-639 [1984].

The present invention still further provides recombinant vectors containing and capable of directing the expression of a DNA encoding a recombinant CEA glycoprotein derivative in a compatible host cell, and host cells containing such vectors. It should also be understood that the nucleotide sequences of the present invention which are to be inserted into a recombinant vectors may include additional nucleotides, which additional nucleotides are not part of the actual structural gene encoding the recombinant CEA glycoprotein derivative of the present invention, as long as the recombinant vectors containing such sequence or fragments are still capable of directing the production of a recombinant CEA glycoprotein derivative in accordance with the present invention in an appropriate host cell.

The insertion of a DNA encoding a recombinant CEA glycoprotein derivative in accordance with the present invention into a cloning vector is easily accomplished when both the DNA and the desired cloning vector have been cut with the same restriction enzyme or enzymes, since complementary DNA termini are thereby produced. If this cannot be accomplished, it may be necessary to modify the cut ends that are produced by digesting back single-stranded DNA to produce blunt ends, or by achieving the same result by filling in the single-stranded termini with an appropriate DNA polymerase. In this way, blunt-end ligation with an enzyme such as T4 DNA ligase may be carried out. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini. Such linkers may comprise specific oligonucleotide sequences that encode restriction site recognition sequences. The cleaved vector and the DNA coding for the recombinant CEA glycoprotein derivative of the present invention may also be modified by homopolymeric tailing (see Morrow J.F., "Recombinant DNA Techniques", Methods in Enzymology 68, 3-24 [1979]).

Many of the cloning vectors known to the person skilled in the art may be used for preparing the recombinant vectors in accordance with the present invention. Such cloning vectors comprise one or more marker activities that may be used to select for desired transformants. Examples for such marker activities are e.g. neomycin resistance (see Example below), geneticin resistance and methotrexate resistance.

It should be understood that there are many ways to insert a DNA encoding the recombinant CEA glycoprotein derivative of the present invention into a cloning vector. What is essential in this respect is that the recombinant vector is capable of directing the production of the recombinant CEA glycoprotein derivative in an appropriate host cell.

The recombinant vectors comprising a DNA having a nucleotide sequence encoding a recombinant CEA glycoprotein derivative of the present invention may be prepared by:

- (a) inserting a DNA having a nucleotide sequence encoding the recombinant CEA glycoprotein derivative into a vector;
- (b) replicating the said vector in a host cell; and
- (c) isolating the recombinant vector from the host cell.

The selection of an appropriate host cell is affected by a number of factors known in the art. These factors include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the recombinant vectors, capability to secrete the desired protein, ease of recovery of the desired CEA glycoprotein, expression characteristics, biosafety and costs. A balance of these factors must be considered, and it must be understood that not all hosts will be equally effective for expression of a particular recombinant DNA molecule. The preferred host cells for producing the recombinant CEA glycoprotein of the

present invention are CEA-negative cells. Examples for such cells are human or rat colon carcinoma cells, e.g. the subclone CO115⁻ from the human colon carcinoma cell line CO115 described by Mach et al. (Mach J. -P., Cartel S., Merenda C., Sordat B. and Cerottini J.-C., "In vivo localization of radiolabelled antibodies to carcinoembryonic antigen in human colon carcinoma grafted into nude mice", *Nature* 248,

- 5 704-706 [1974]; see also Carrel S., Sordat B. and Merenda C., "Establishment of a cell line (Co-115) from a human colon carcinoma transplanted into nude mice", *Cancer Res.* 36, 3978-3984 [1976]) and the rat colon carcinoma cell line PROb described by Caillard et al. (Caillard A., Martin M. S., Michel M. F. and Martin F., "Interaction between two cellular subpopulations of a rat colonic carcinoma when inoculated to the syngeneic host", *Int. J. Cancer* 36, 273-279 [1985]). The said subclone CO115⁻ was shown by fluorescent
- 10 activated cell sorting using a panel of directly fluorescinated anti-CEA monoclonal antibodies, to express no CEA and no CEA-crossreactive antigens at the cell surface. It was also shown by enzyme linked immunoassay that the culture medium from the untransfected CO115⁻ subclone contains no CEA or CEA-crossreacting antigens which may interfere in the assay.

Various methods for introducing a foreign DNA fragment into a cell are known to the person skilled in the art. Examples for such methods are microinjection, electroporation, transfection and infection with a viral vector. The preferred method for inserting a recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention into a cell is the well-known calcium phosphate method (originally described by Graham F.L. and Van der Eb A.J., "A new technique for the assay of infectivity of human adenovirus 5 DNA", *Virology* 54, 456-467 [1973]). Geneticin-resistant transfecants can be screened 15 for CEA secretion in the supernatant by an ELISA test using anti-CEA antibodies. Once a transformed host cell is produced, the cell population may be amplified in culture a culture medium comprising the necessary nutrients under conditions suitable for the growth of the cell population and/or under conditions suitable for high secretion of the recombinant DNA leading to the production of high amounts of the recombinant CEA glycoprotein derivative of the present invention.

20 25 The secreted recombinant CEA glycoprotein derivative of the present invention is secreted into the cell culture medium from which it can be isolated by first removing the cells and cellular debris by low speed centrifugation. The supernatant containing the recombinant CEA glycoprotein derivative of the present invention obtained in this way may then be concentrated by ultrafiltration. Initial separation of the recombinant CEA glycoprotein derivative from the supernatant may be performed by precipitation with salts 30 such as sodium or ammonium sulfate, by ultrafiltration or by other methods well known to those skilled in the art. Further purification can be accomplished by conventional protein purification techniques including but not limited to gel filtration, ion-exchange chromatography, preparative disc-gel or curtain electrophoresis, isoelectric focusing, low temperature organic solvent fractionation, or countercurrent distribution. Purification can also be carried out by immunoaffinity chromatography.

35 30 Thus, the present invention provides also a method for producing the recombinant CEA glycoprotein derivative defined above, which method comprises:

- (a) culturing a host cell containing a recombinant vector comprising a DNA having a nucleotide sequence 40 encoding the said recombinant CEA glycoprotein derivative, such as the DNA having the nucleotide sequence [SEQ ID NO: 2] or an equivalent sequence thereof, under conditions in which the DNA is expressed; and
- (b) isolating the recombinant CEA glycoprotein derivative produced by the host cell from the culture.

The preferred method for the construction of the recombinant cDNA encoding the CEA fragment of the present invention can be summarized as follows:

- 45 - In the first step, a suitable vector comprising a cDNA encoding the full length CEA protein, such as the Bluescript™ vector described in the Example below, is digested at a unique site with the Bsu36I endonuclease. The cleaved vector is then dephosphorylated and further digested at a unique site with the XbaI endonuclease to generate two fragments of 0.8 and 4.6 Kb (1 Kb = 1'000 base pairs) which are isolated.
- 50 - In the second step, the 4.6 Kb fragment, containing vector DNA and the 5' part of the CEA cDNA, is ligated into a XbaI site with a synthetic oligonucleotide duplex containing the last 43 base pairs (b.p.) of the third CEA repeat downstream from the EaeI site to the codon of the last amino acid found in the mature protein and further containing a TAG stop codon and a XbaI sticky end.
- 55 - The third step consists of cleaving the 0.8 Kb fragment referred to above at its unique EaeI site. This resulted in two fragments of about 0.4 Kb each, one fragment consisting of the missing part of the third CEA repeat which was to be retained in the final recombinant cDNA and the other fragment consisting of the hydrophobic tail which should be deleted.
- In the fourth step the vector is circularized. Of the two 0.4 Kb fragments obtained in the previous step, only the one containing the third CEA repeat has the proper sticky ends, viz. Bsu36I and XbaI, for the

double ligation and circularization of the 4.6 Kb fragment joined to the synthetic duplex mentioned above. The resulting construct may be amplified in a suitable vector such as in a Bluescript™ vector. The correct construction of the vector can be checked by restriction analysis using *Sty*I and *Eae*I endonucleases. The recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention can then be recloned into a vector suitable for the expression of a cDNA in a eukaryotic cell. An example for such a vector is the eukaryotic expression vector pRc/CMV referred to in the Example below. The vector pRc/CMV is designed for high level stable expression of inserted genes under the control of the constitutive CMV promoter. The vector comprises also the bovine growth hormone polyadenylation signal and a neomycin resistance gene which is expressed from the SV40 early promoter. The correctness of the final construct can be verified by restriction analysis. The correctness of the new 3' end of the recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention can be confirmed by sequencing.

The vector comprising the recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention and the neomycin-resistance gene are preferably introduced by using the calcium phosphate method transfection method into a CEA-negative cell, such as e.g. the subclone from the human colon carcinoma cell line CO115 (Mach J. -P. et al. [1974], supra; Carrel S. et al. [1976], supra) or the rat colon carcinoma cell line PROb (Caignard A. et al. [1985], supra).

Depending on the host cell used for inserting the recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention, the transformed host cells were found to shed about 50 to 100 times more CEA than host cells transformed with a cDNA encoding the full length CEA protein. Thus, e.g. the subclones from the human colon carcinoma cell line CO115 transformed with the recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention were found to secrete about 7.7 to 13.6 micrograms CEA/10⁶ cells/72 h. Under the same conditions, non-transfected human colon carcinoma cells known to have a high level of CEA expression shed about 50 to 300 times less CEA. More precisely the cell line CO112 (Mach J.P. et al. [1974], supra) shed about 0.045 micrograms CEA/10⁶ cells/72 h and the cell line LS174T (Rutzky L.P., Kaye C.I., Siciliano M.J., Chao M. and Kahan B.D., "Longitudinal karyotype and genetic signature analysis of cultured human colon adenocarcinoma cell lines LS180 and LS174T", *Cancer Res.* 40, 1443-1448 [1980]) shed 0.128 micrograms CEA/10⁶ cells/72 h).

The PROb rat carcinoma clones transfected with the recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention secreted from 0.61 to 0.99 micrograms CEA/10⁶ cells/72 h. Selected clones from the same PROb cells transfected with full-length CEA-cDNA shed a maximum of only 0.015 micrograms CEA/10⁶ cells/72 h (Pèlegrein A., Terskikh A., Hayoz D., Chalandon Y., Olsson N. O., Folli S., Buchegger F., Kromer B., Schwarz K., Martin M., Martin F. and Mach J. -P., "Human carcinoembryonic antigen cDNA expressed in rat carcinoma cells can function as target antigen for tumor localization of antibodies in nude rats and as rejection antigen in syngeneic rats", *Int. J. Cancer* 52, 110-119 [1992]). Thus, the transfection of rat colon carcinoma PROb with a CEA-cDNA lacking the C-terminal domain resulted in a 50 fold higher level of CEA secretion compared to clones from the same cell line transfected with full-length CEA cDNA.

The above results show that there is a difference between the amount of recombinant CEA glycoprotein derivative of the present invention (rCEA) secreted by human cells and rat cells. It has been proposed earlier that both transcriptional and post-transcriptional control mechanisms regulate CEA gene expression in colon carcinomas (Hauck W. and Stanners C. P., "Control of carcinoembryonic antigen gene family expression in a differentiating colon carcinoma cell line, Caco-2", *Cancer Res.* 51, 3526-3533 [1991]). In view of the identity of the DNA constructs used for transfection this lower secretion rate of rCEA may be attributed to the differences in post-transcriptional control between the two species.

The size of the CEA expressed by the transfected cells can be analyzed by Western blotting (Towbin, H., Staehelin, T. and Gordon J., "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications", *Proc. Natl. Acad. Sci. USA* 76, 4350-4354 [1979]). Such an analysis is performed as follows: Cell culture supernatants are electrophoretically separated on a SDS-polyacrylamide gel, preferably a 7.5-15% linear gradient SDS-polyacrylamide gel. Then an immunoblot is performed using antibodies recognizing a major epitope on the CEA protein. Preferably a pool of ¹²⁵I-labeled anti-CEA monoclonal antibodies (MAbs) is used for such purpose. In a typical example the rCEA cDNA-transfected-CO115 human colon carcinoma exemplified below produced a rCEA of about 200 kDa. This corresponds to the size of the CEA protein produced by human colon carcinoma LS174T. When transfected into the PROb rat colon carcinoma, the same rCEA cDNA produced a rCEA with a lower molecular weight (viz. about 144 kDa) as did the full-length CEA cDNA transfected into the PROb rat carcinoma cells (Pèlegrein et al. [1992], supra).

Epitope characterization of ^{125}I -labeled, purified rCEA produced by the host cells transformed with the recombinant cDNA encoding the recombinant CEA glycoprotein derivative of the present invention can be performed by testing the binding of the said rCEA to different anti-CEA MAbs coupled to SepharoseTM. In typical experiments using human colon carcinoma CO115 cells transformed with a recombinant cDNA 5 encoding the recombinant CEA glycoprotein derivative of the present invention the five MAbs GOLD 1 to 5 directed against the 5 major epitopes on the CEA molecule (Hammarstrom et al. [1989], supra), binding values ranging from 65% to 88% were found (see Example below). Such binding values compare favorably with those obtained with ^{125}I -labeled CEA purified from a human tumor.

Thus the present invention shows that transfection into human and rat carcinoma cells of a recombinant 10 CEA cDNA clone from which the region coding for the hydrophobic C-terminal domain has been deleted, results in an abundant secretion of fully antigenic rCEA molecules into the medium. As mentioned above CEA is normally anchored to the cell membrane by a phosphatidylinositol-glycan (PI-G) and is only shed into the medium of cultured cells or in the serum of carcinoma patients probably after the cleavage from the membrane by PI-specific phospholipases. The recombinant CEA glycoprotein derivative of the present 15 invention is lacking the C-terminal hydrophobic tail. Therefore, it cannot be PI-G anchored and is directly secreted into the extracellular space. It was found that the lack of the hydrophobic domain in the CEA glycoprotein does not affect the transport of CEA towards the cell surface in either human or rat carcinomas, but only prevents its anchoring to the cell surface. The recombinant CEA glycoprotein derivative of the present invention is recognized by the five well characterized epitopes GOLD 1 - 5.

20 A Western blot analysis has shown, that surprisingly the rCEA secreted from the transfected rat colon carcinoma has a lower molecular weight (about 144 kDa) than a reference CEA isolated from a human colon carcinoma (about 200 kDa). This is in agreement with the observation that the CEA produced by rat colon carcinoma cells transfected with full-length CEA cDNA has also the same "lower" molecular weight after 25 cleavage by PI-PLC (Pèlegrin et al. [1992], supra). CEA molecules with abnormal molecular weights expressed in heterologous cells transfected with a full length CEA gene have been observed in the case of mouse L-cells and chinese hamster ovary cells (Hefta et al. 1990). The molecules identified on L-cells transfected with total human DNA had a lower molecular weight (150 kDa) than those identified on transfected hamster cells (180 kDa). Incomplete glycosylation seems to be responsible for the smaller 30 molecular weight of the CEA molecules expressed by rat colon carcinoma cells transfected with either full length CEA cDNA or truncated CEA cDNA.

The present invention can be more readily understood by reference to the following Example and the Figures the contents of which Figures are as follows:

Figure 1: Schematic outline of the construction of an exemplary recombinant truncated CEA cDNA in accordance with the present invention. The black box represents the deleted hydrophobic 35 domain. Phosphorylated ends are marked by P.

Figure 2: CEA secretion by different clones and control cell lines. CO115, a CEA-negative clone of a human colon carcinoma cell line; 2C2, 1D6, 2B12, truncated CEA-cDNA CO115 derived transfectants; CO112, LS174T, high CEA-expressing human colon carcinoma cell lines; PROb, a rat colon carcinoma cell line; 1H5, 1A8, 1G7, truncated CEA-cDNA PROb derived 40 transfectants; 3G7/2C11, full-length CEA-cDNA PROb derived transfectants (Pèlegrin et al. [1991], supra).

Figure 3: Western blot analysis of CEA from different clones and control cell lines. Cell culture supernatants were run either directly for the CEA-secreting clones or after treatment of the 45 cells with PI-PLC for the other cells. LS174T, CEA-expressing human colon carcinoma cell line; CO115, CEA-negative clone of human colon carcinoma cell line; 2C2, 1D6, 2B12, truncated CEA-cDNA CO115 derived transfectants; PROb, a rat colon carcinoma cell line; 3G7/2C11, full-length CEA-cDNA PROb derived transfectants (Pèlegrin et al. [1992], supra); 1H5, 1A8, 1G7, truncated CEA-cDNA PROb derived transfectants.

Figure 4: Binding of ^{125}I -control CEA (□) and ^{125}I -rCEA (■) to immobilized MAbs directed against the 50 different CEA epitopes. About 5 ng CEA were incubated for 16 hours at 25°C with 5 micrograms of each of the 5 anti-CEA MAbs covalently coupled to SepharoseTM.

EXAMPLE

55 Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids, and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively. Furthermore, unless otherwise specified, the suppliers of reagents including the full-length CEA cDNA, as well as the instruments mentioned below are not meant to be mandatory. The skilled person is in a position to select similar

reagents or instruments from other suppliers.

Deletion of the region coding for the hydrophobic tail of CEA

5 The Bluescript™ (KS+) vector containing full-length CEA cDNA between unique HindIII and XbaI sites (Zimmermann W., Weber B., Ortlieb B., Rudert F., Schempp W., Fiebig H., Shively J. E., von Kleist S. and Thompson J. A., "Chromosomal localization of the carcinoembryonic antigen gene family and differential expression in various tumors", *Cancer Res.* 48, 2550-2554 [1988]; Pèlegrin et al. [1992], supra)) was digested with endonuclease Bsu36I (Boehringer, Mannheim, Germany) and dephosphorylated with alkaline phosphatase (Boehringer). A second digestion with XbaI (Pharmacia, Uppsala, Sweden) produced two fragments of 0.8 Kb and 4.6 Kb which were separated and electroeluted from a 1% agarose gel (see Fig 1, step 1).

10 Two oligonucleotides (46 b. each), encoding both strands of the last 43 basepairs of the third CEA repeat, viz. JPM1

15

5'-GGCCGCAATAATTCCATAGTCAAGAGCATCACAGTCTCTGCATAGT - 3'
[SEQ ID NO: 3]

20 and JPM2

25

5'-CTAGACTATGCAGAGACTGTGATGCTCTTGACTATGGAATTATTGC - 3'
[SEQ ID NO: 4],

respectively were synthesized on a commercial DNA synthesizer. The oligonucleotides were purified by passage through a Sephadex™ NAP-25 column (Pharmacia), lyophilized and dissolved in 10 mM Tris-HCl buffer pH 7.6 containing 0.1 mM EDTA.

30 After gel purification and annealing, the synthetic oligonucleotides form a synthetic duplex encompassing the 43 b.p. of the third CEA repeat downstream from the EaeI to the codon of the last amino acid found in the mature protein. This was followed by a TAG stop codon and by a XbaI sticky end, which forms the 3' end, while the 5' end had a EaeI sticky end.

The duplex was ligated into the XbaI site with the large 4.6 Kb fragment by overnight incubation at 8°C in 1mM ATP (see Fig.1, step 2). The reaction mixture was separated on a 1% agarose gel in order to purify the 4.6 fragment ligated to the synthetic duplex from the excess of the free duplex form.

35 The 0.8 Kb fragment was digested with EaeI (Boehringer) (see Fig.1, step 3) and the DNA mixture was ligated with the 4.6 Kb fragment joined to the synthetic duplex (see Fig.1, step 4). After phosphorylation of the Bsu36I site with polynucleotide kinase 4 (Pharmacia), the construct was circularized and transfected into the E.coli strain XL1-blue (Stratagene Cloning Systems, La Jolla, California). Individual clones were analyzed by restriction with StyI and EaeI endonucleases. DNA from selected clones was amplified and isolated. Recombinant CEA cDNA was cut out by double digestion with HindIII and XbaI endonucleases and cloned into a pRc/CMV expression vector (Invitrogen, San Diego, California).

40 Individual clones were further controlled with the restriction endonucleases StyI and EaeI. DNA from selected clones was amplified, isolated and about 200 nucleotides from the 3' end of the recombinant CEA cDNA, including the 46 b.p. region formed by the synthetic duplex, were sequenced using T7 and SP6 primer with a USB Sequencing kit (USB, Cleveland, Ohio).

Cell cultures and transfections

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The human colon carcinoma cell line CO115 was established according to known procedures (Mach et al. [1974], supra; Carrel et al. [1976], supra). The rat colon carcinoma cell line DHD/K12/TRb, referred to as PROb, is a selected subclone derived from a cell line established from a transplantable colon adenocarcinoma induced by 1,2-dimethylhydrazine in a syngeneic BDIX rat (Martin F., Caignard A., Jeannin J. F., Leclerc A. and Martin M., "Selection by trypsin of two sublines of rat colon cancer cells forming progressive or regressive tumors", *Int. J. Cancer.* 32, 623-627 [1983]). The PROb subclone has been shown to induce progressive tumors in rats of the Berlin Druckrey IX/Orl (BDIX) strain (Caignard et al. [1985], supra). The human and rat cell lines were maintained in RPMI 1640 and Dulbecco F12 medium, respectively,

supplemented with 10% fetal calf serum (FCS). Three micrograms of DNA were precipitated with calcium phosphate (Mammalian Transfection Kit, Stratagene, La Jolla, California) and incubated for 16 hours with about 3×10^6 nonconfluent adherent carcinoma cells in 10 ml of culture medium with 10% FCS. The medium was removed and 10 ml of fresh culture medium were then added. After a further 24-hours 5 incubation, the cells were harvested, distributed into four 96-well microtiter plates and grown for 24 hours before adding the neomycin analog G418 (Gibco, Paisley, Scotland) at a concentration of 200 micrograms/ml. The supernatants from each well were screened by ELISA (Buchegger F., Mettraux C., Accolla R. S., Carrel S. and Mach J. -P., "Sandwich enzyme immunoassay using three monoclonal antibodies against different epitopes of carcinoembryonic antigen (CEA)", *Immunol. Lett.* 5, 85-91 [1982]) for CEA secretion.

10

Monoclonal antibodies

MAb B93, 35, B17, and CE25 are specific for CEA; they do not bind to crossreacting antigens nor to granulocytes (Buchegger F., Pèlegrin A., Delaloye B., Bischof-Delaloye A. and Mach J. -P., " ^{131}I labeled F-15 (ab')2 fragments are more efficient and less toxic than intact anti-CEA antibodies in radioimmunotherapy of large human colon carcinoma grafted in nude mice", *J. Nucl. Med.* 31, 1035-1044 [1984]). MAb 192 is an anti-CEA antibody which cross-reacts with non-specific crossreacting antigen (NCA) (Buchegger F., Schreyer M., Carrel S. and Mach J. -P., "Monoclonal antibodies identify a CEA crossreacting antigen of 95 kD (NCA-95) distinct in antigenicity and tissue distribution from the previously described NCA of 55 kD", 20 *Int. J. Cancer* 33, 643-649 [1984]). Each of the five Mabs (B93, 35, B17, CE25 and 192) reacts specifically with one of the recently identified Gold 1-5 epitopes of the CEA molecule (Hammarstrom et al. [1989], supra).

25

Assay for CEA production

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An equal number of cells (5×10^5) from CEA cDNA transfected human or rat carcinoma clones or from untransfected human colon carcinomas was added to each well of a 24-well culture plate (Falcon, Becton Dickinson, Oxnard, USA) in 10% FCS RPMI medium. After 18 hours, the complete medium was replaced by 1 ml serum-free medium, which markedly decreases cell proliferation without inhibiting CEA secretion. 30 The supernatants were harvested following a further 72 hours incubation. The amount of CEA in the supernatants was determined by an enzyme linked immunoabsorbant assay (ELISA) using 3 anti-CEA MAbs (Buchegger et al. [1982], supra).

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CEA purification and labeling with ^{125}I

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rCEA was affinity purified from serum-free culture supernatant on an immunoabsorbant column consisting of MAb B17 coupled to Sepharose™. Batches of 50 ml serum-free culture supernatant were applied at a rate of 2 ml/hour on a 2 ml Sepharose™ column containing 4 mg of B17 MAb. The bound CEA was eluted from the column with 3 M ammonium thiocyanate in H_2O and immediately dialyzed against 0.1 M Tris buffer, pH 7.4.

40

CEA was extracted from liver metastases using the perchloric acid method (Krupey J., Wilson T., Freedman S. O. and Gold P., "The preparation of purified carcinoembryonic antigen of the human digestive system from large quantities of tumour tissue", *Immunochem.* 9, 617-622 [1972]; Fritsche R. and Mach J. P., "Isolation and characterization of carcinoembryonic antigen (CEA) extracted from normal human colon mucosa", *Immunochem.* 14, 119-127 [1977]). Briefly, one volume of tissue was first homogenized in 3 volumes of 0.03 M phosphate buffer, pH 7.0 at 4 °C for 10 minutes in a Sorvall Omnimixer (Sorvall, Newton, CT, USA) at 8,000 rpm. The crude homogenate was extracted with 0.6 M perchloric acid for 20 minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was dialyzed against deionized water, lyophilized, dissolved in Tris buffer and purified by gel filtration on a Sephadex™ G-200 column followed by a Sepharose™ 6B column.

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Batches of 20 micrograms purified CEA and rCEA were labeled with 1 mCi ^{125}I using the chloramine T method. The iodine incorporation was about 30-40%. ^{125}I -labeled CEA and rCEA were further purified by gel filtration on a Sephadex™ G-200 column.

55

Epitope characterization

The different epitopes of the CEA molecule were analyzed in a direct binding assay. About 5 ng ^{125}I -CEA were incubated for 16 hours at 25 °C with 5 micrograms of each of the 5 anti-CEA MAbs coupled to

CNBr-Sepharose™ (Pharmacia). The percentage of specific binding was determined by measuring the radioactivity bound to the MAb. The non-specific binding of ^{125}I -CEA was determined by similar incubation with an irrelevant IgG coupled to Sepharose™.

5 Western blot analysis

Cell culture supernatants from selected transfected clones secreting rCEA were analyzed by Western blot analysis without further treatment. Control untransfected human colon carcinoma cells ($5\text{-}10 \times 10^6$) synthesizing membrane-bound CEA were treated with 0.5-1.0 unit of phosphatidylinositol-specific 10 phospholipase C (PI-PLC) (Boehringer Mannheim, Germany) for 1 hour at 37°C in RPMI medium containing 1 mg/ml BSA and 20 mM EDTA.

Samples of cell culture supernatants containing about 100 ng CEA were run on a 7.5-15% linear gradient SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). Biotinylated 15 SDS-PAGE standards (Bio-Rad, Richmond, CA) were used to determine molecular weights. Membranes were incubated overnight with a pool of 4 ^{125}I -labeled anti-CEA MAbs (35, CE25, B93 and B17) and ^{125}I -labeled avidin at 4°C and then subjected to autoradiography.

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SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: CEA Derivatives

(iii) NUMBER OF SEQUENCES: 4

20 (iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

25 (vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: EP 93810214.2
(B) FILING DATE: 25-MAR-1993

30 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 642 amino acids
(B) TYPE: amino acid
35 (C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

40 (iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
45 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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1 5 10 15

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					20				25					30		
5	Trp	Tyr	Lys	Gly	Glu	Arg	Val	Asp	Gly	Asn	Arg	Gln	Ile	Ile	Gly	Tyr
					35			40					45			
	Val	Ile	Gly	Thr	Gln	Gln	Ala	Thr	Pro	Gly	Pro	Ala	Tyr	Ser	Gly	Arg
					50			55				60				
10	Glu	Ile	Ile	Tyr	Pro	Asn	Ala	Ser	Leu	Leu	Ile	Gln	Asn	Ile	Ile	Gln
					65			70			75		80			
	Asn	Asp	Thr	Gly	Phe	Tyr	Thr	Leu	His	Val	Ile	Lys	Ser	Asp	Leu	Val
					85				90			95				
15	Asn	Glu	Glu	Ala	Thr	Gly	Gln	Phe	Arg	Val	Tyr	Pro	Glu	Leu	Pro	Lys
					100				105			110				
20	Pro	Ser	Ile	Ser	Ser	Asn	Asn	Ser	Lys	Pro	Val	Glu	Asp	Lys	Asp	Ala
					115			120				125				
	Val	Ala	Phe	Thr	Cys	Glu	Pro	Glu	Thr	Gln	Asp	Ala	Thr	Tyr	Leu	Trp
					130			135			140					
25	Trp	Val	Asn	Asn	Gln	Ser	Leu	Pro	Val	Ser	Pro	Arg	Leu	Gln	Leu	Ser
					145			150			155		160			
	Asn	Gly	Asn	Arg	Thr	Leu	Thr	Leu	Phe	Asn	Val	Thr	Arg	Asn	Asp	Thr
					165				170			175				
30	Ala	Ser	Tyr	Lys	Cys	Glu	Thr	Gln	Asn	Pro	Val	Ser	Ala	Arg	Arg	Ser
					180			185				190				
	Asp	Ser	Val	Ile	Leu	Asn	Val	Leu	Tyr	Gly	Pro	Asp	Ala	Pro	Thr	Ile
					195			200			205					
35	Ser	Pro	Leu	Asn	Thr	Ser	Tyr	Arg	Ser	Gly	Glu	Asn	Leu	Asn	Leu	Ser
					210			215			220					
40	Cys	His	Ala	Ala	Ser	Asn	Pro	Pro	Ala	Gln	Tyr	Ser	Trp	Phe	Val	Asn
					225			230			235		240			
	Gly	Thr	Phe	Gln	Gln	Ser	Thr	Gln	Glu	Leu	Phe	Ile	Pro	Asn	Ile	Thr
					245				250			255				
45	Val	Asn	Asn	Ser	Gly	Ser	Tyr	Thr	Cys	Gln	Ala	His	Asn	Ser	Asp	Thr
					260			265			270					
	Gly	Leu	Asn	Arg	Thr	Thr	Val	Thr	Thr	Ile	Thr	Val	Tyr	Ala	Glu	Pro
					275			280			285					
50	Pro	Lys	Pro	Phe	Ile	Thr	Ser	Asn	Asn	Ser	Asn	Pro	Val	Glu	Asp	Glu
					290			295			300					

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	Asp Ala Val Ala Leu Thr Cys Glu Pro Glu Ile Gln Asn Thr Thr Tyr			
305	310	315	320	
5	Leu Trp Trp Val Asn Asn Gln Ser Leu Pro Val Ser Pro Arg Leu Gln			
	325	330	335	
	Leu Ser Asn Asp Asn Arg Thr Leu Thr Leu Leu Ser Val Thr Arg Asn			
	340	345	350	
10	Asp Val Gly Pro Tyr Glu Cys Gly Ile Gln Asn Glu Leu Ser Val Asp			
	355	360	365	
	His Ser Asp Pro Val Ile Leu Asn Val Leu Tyr Gly Pro Asp Asp Pro			
15	370	375	380	
	Thr Ile Ser Pro Ser Tyr Thr Tyr Tyr Arg Pro Gly Val Asn Leu Ser			
	385	390	395	400
20	Leu Ser Cys His Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser Trp Leu			
	405	410	415	
	Ile Asp Gly Asn Ile Gln Gln His Thr Gln Glu Leu Phe Ile Ser Asn			
	420	425	430	
25	Ile Thr Glu Lys Asn Ser Gly Leu Tyr Thr Cys Gln Ala Asn Asn Ser			
	435	440	445	
	Ala Ser Gly His Ser Arg Thr Thr Val Lys Thr Ile Thr Val Ser Ala			
	450	455	460	
30	Glu Leu Pro Lys Pro Ser Ile Ser Ser Asn Asn Ser Lys Pro Val Glu			
	465	470	475	480
	Asp Lys Asp Ala Val Ala Phe Thr Cys Glu Pro Glu Ala Gln Asn Thr			
	485	490	495	
35	Thr Tyr Leu Trp Trp Val Asn Gly Gln Ser Leu Pro Val Ser Pro Arg			
	500	505	510	
	Leu Gln Leu Ser Asn Gly Asn Arg Thr Leu Thr Leu Phe Asn Val Thr			
	515	520	525	
40	Arg Asn Asp Ala Arg Ala Tyr Val Cys Gly Ile Gln Asn Ser Val Ser			
	530	535	540	
	Ala Asn Arg Ser Asp Pro Val Thr Leu Asp Val Leu Tyr Gly Pro Asp			
45	545	550	555	560
	Thr Pro Ile Ile Ser Pro Pro Asp Ser Ser Tyr Leu Ser Gly Ala Asn			
	565	570	575	
50	Leu Asn Leu Ser Cys His Ser Ala Ser Asn Pro Ser Pro Gln Tyr Ser			
	580	585	590	

Trp Arg Ile Asn Gly Ile Pro Gln Gln His Thr Gln Val Leu Phe Ile
 595 600 605
 Ala Lys Ile Thr Pro Asn Asn Asn Gly Thr Tyr Ala Cys Phe Val Ser
 610 615 620
 5 Asn Leu Ala Thr Gly Arg Asn Asn Ser Ile Val Lys Ser Ile Thr Val
 625 630 635 640
 Ser Ala

10 (2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2031 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGGAGTCTC CCTCGGCCCC TCCCCACAGA TGGTGCATCC CCTGGCAGAG GCTCCTGCTC	60
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CATCTTTTG GCTACAGCTG GTACAAAGGT GAAAGACTGG ATGGCAACCG TCAAATTATA	240
GGATATGTAA TAGGAACTCA ACAAGCTACC CCAGGGCCCG CATAACAGTGG TCGAGAGATA	300
35 ATATAACCCA ATGCATCCCT GCTGATCCAG AACATCATCC AGAATGACAC AGGATTCTAC	360
ACCCTACACG TCATAAAAGTC AGATCTTGTG AATGAAGAAG CAACTGGCCA GTTCCGGTA	420
TACCCGGAGC TGCCCAAGCC CTCCATCTCC AGCAACAACCT CCAAACCCGT GGAGGACAAG	480
40 GATGCTGTGG CCTTCACCTG TGAACCTGAG ACTCAGGACG CAACCTACCT GTGGTGGTA	540
AACAATCAGA GCCTCCCGGT CAGTCCCAGG CTGCAGCTGT CCAATGGCAA CAGGACCCCTC	600
ACTCTATTCA ATGTACAAG AAATGACACA GCAAGCTACA AATGTGAAAC CCAGAACCCA	660
45 GTGAGTGCCA GGCGCAGTGA TTCAGTCATC CTGAATGTCC TCTATGGCCC GGATGCC	720

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	ACCATTTCCC CTCTAACAC ATCTTACAGA TCAGGGAAA ATCTGAAACCT CTCCTGCCAC	780
	GCAGCCTCTA ACCCACCTGC ACAGTACTCT TGGTTTGTCATGGGACTTT CCAGCAATCC	840
5	ACCCAAGAGC TCTTTATCCC CAACATCACT GTGAATAATA GTGGATCCTA TACGTGCCAA	900
	GCCCCATAACT CAGACACTGG CCTCAATAGG ACCACAGTCA CGACGATCAC AGTCTATGCA	960
	GAGCCACCCA AACCCCTCAT CACCAGCAAC AACTCCAACC CCGTGGAGGA TGAGGATGCT	1020
10	GTAGCCTTAA CCTGTGAACC TGAGATTCAAG AACACAACCT ACCTGTGGTG GGTAAATAAT	1080
	CAGAGCCTCC CGGTCACTGCAG CTGTCCAATG ACAACAGGAC CCTCACTCTA	1140
	CTCAGTGTCA CAAGGAATGA TGTAGGACCC TATGAGTGTG GAATCCAGAA CGAATTAAGT	1200
15	GTTGACCACA GCGACCCAGT CATCCTGAAT GTCCTCTATG GCCCAGACGA CCCCACCATT	1260
	TCCCCCTCAT ACACCTATTAA CCGTCCAGGG GTGAACCTCA GCCTCTCCTG CCATGCAGCC	1320
	TCTAACCCAC CTGCACAGTA TTCTTGGCTG ATTGATGGGA ACATCCAGCA ACACACACAA	1380
20	GAGCTTTA TCTCCAACAT CACTGAGAAG AACAGCGGAC TCTATACCTG CCAGGCCAAT	1440
	AACTCAGCCA GTGGCCACAG CAGGACTACA GTCAAGACAA TCACAGTCTC TGCGGAGCTG	1500
	CCCAAGCCCT CCATCTCCAG CAACAACTCC AAACCCGTGG AGGACAAGGA TGCTGTGGCC	1560
25	TTCACCTGTG AACCTGAGGC TCAGAACACA ACCTACCTGT GGTGGGTAAA TGGTCAGAGC	1620
	CTCCCAGTCA GTCCCAGGCT GCAGCTGTCC AATGGCAACA GGACCCCTCAC TCTATTCAAT	1680
	GTCACAAAGAA ATGACGCAAG AGCCTATGTA TGTGGAATCC AGAACTCAGT GAGTGCACAA	1740
30	CGCAGTGACC CAGTCACCCCT GGATGTCCTC TATGGGCCGG ACACCCCCAT CATTCCCCC	1800
	CCAGACTCGT CTTACCTTTC GGGAGCGAAC CTCAACCTCT CCTGCCACTC GGCCTCTAAC	1860
	CCATCCCCGC AGTATTCTTG GCGTATCAAT GGGATACCGC AGAACACAC ACAAGTTCTC	1920
35	TTTATGCCA AAATCACGCC AAATAATAAC GGGACCTATG CCTGTTTGT CTCTAACTTG	1980
	GCTACTGGCC GCAATAATTCAAG AGCATCACAG TCTCTGCATA G	2031

(2) INFORMATION FOR SEQ ID NO: 3:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 46 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

10 GGCCGCAATA ATTCCATAGT CAAGAGCATC ACAGTCTCTG CATACT

46

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46 base pairs

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTAGACTATG CAGAGACTGT GATGCTCTTG ACTATGGAAT TATTGC

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Claims

35 1. A recombinant CEA glycoprotein derivative characterized in that it is:

(a) free from cross-reactive CEA-like antigens;

(b) antigenically indistinguishable from the soluble form of CEA shed from tumor cells; and

(c) devoid of ethanolamine.

40 2. A recombinant CEA glycoprotein derivative according to claim 1, having the amino acid sequence

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KLTIESTPFN VAEGKEVLLL VHNLPQHLFG YSWYKGERVD GNRQIIGYVI
5 GTQQATPGPA YSGREIIYPN ASLLIQNIIQ NDTGFYTLHV IKSDLVNEEA
TGQFRVYPEL PKPSISSNNS KPVEDKDAVA FTCEPETQDA TYLWWVNNQS
10 LPVSPRLQLS NGNRTLTLFN VTRNDTASYK CETQNPVSAR RSDSVILNVL
YGPDAPTISP LNTSYRSGEN LNLSCHAASN PPAQYSWFVN GTFQQSTQEL
15 FIPNITVNNS GSYTCQAHNS DTGLNRTTVT TITVYAEPPK PFITSNNNSNP
VEDEDAVALT CEPEIQNTTY LWWVNNQSLP VSPRLQLSND NRTLTLLSVT
20 RNDVGPYECG IQNELSVDHS DPVILNVLYG PDDPTISPSY TYYRPGVNLS
LSCHAASNPP AQYSWLIDGN IQQHTQELFI SNITEKNNSGL YTCQANNSAS
25 GHSRTTVKTI TVSAELPKPS ISSNNSKPVE DKDAVAFTCE PEAQNTTYLW
WVNGQSLPVS PRLQLSNGNR TLTLFNVTRN DARAYVCGIQ NSVSANRSDP
30 VTLDVLYGPD TPIISPPDSS YLSGANLNLS CHSASNPSPQ YSWRINGIPQ
QHTQVLFIAK ITPNNNGTYA CFVSNLATGR NNSIVKSITV SA **SEQ ID NO: 1]**

35 or an allelic variant or functional equivalent thereof.

3. A DNA encoding a recombinant CEA glycoprotein derivative according to claim 1 or 2.
4. A DNA encoding a recombinant CEA glycoprotein derivative according to claim 1 or 2 having all or part
40 of the nucleotide sequence

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ATGGAGTCTC CCTCGGCCCC TCCCCACAGA TGGTGCATCC CCTGGCAGAG
 5 GCTCCTGCTC ACAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCACTG
 CCAAGCTCAC TATTGAATCC ACGCCGTTCA ATGTCGCAGA GGGGAAGGAG
 10 GTGCTTCTAC TTGTCCACAA TCTGCCAG CATCTTTG GCTACAGCTG
 GTACAAAGGT GAAAGAGTGG ATGGCAACCG TCAAATTATA GGATATGTAA
 15 TAGGAACTCA ACAAGCTACC CCAGGGCCCG CATAACAGTGG TCGAGAGATA
 ATATAACCCA ATGCATCCCT GCTGATCCAG AACATCATCC AGAATGACAC
 20 AGGATTCTAC ACCCTACACG TCATAAAAGTC AGATCTTGTG AATGAAGAAG
 CAACTGGCCA GTTCCGGGTA TACCCGGAGC TGCCCAAGCC CTCCATCTCC
 25 AGCAACAAC CCAAACCCGT GGAGGACAAG GATGCTGTGG CCTTCACCTG
 TGAACCTGAG ACTCAGGACG CAACCTACCT GTGGTGGGTA AACAAATCAGA
 30 GCCTCCCGGT CAGTCCCAGG CTGCAGCTGT CCAATGGCAA CAGGACCCTC
 ACTCTATTCA ATGTCACAAG AAATGACACA GCAAGCTACA AATGTGAAAC
 35 CCAGAACCCA GTGAGTGCCA GGCGCAGTGA TTCAGTCATC CTGAATGTCC
 TCTATGGCCC GGATGCCCTT ACCATTTCCC CTCTAACAC ATCTTACAGA
 40 TCAGGGAAA ATCTGAACCT CTCCTGCCAC GCAGCCTCTA ACCCACCTGC
 ACAGTACTCT TGTTTGTCATGGACTTT CCAGCAATCC ACCCAAGAGC
 45 TCTTTATCCC CAACATCACT GTGAATAATA GTGGATCCTA TACGTGCCAA
 GCCCATAACT CAGACACTGG CCTCAATAGG ACCACAGTCA CGACGATCAC

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AGTCTATGCA GAGCCACCCA AACCCCTTCAT CACCAGCAAC AACTCCAACC
 5 CCGTGGAGGA TGAGGGATGCT GTAGCCTTAA CCTGTGAACC TGAGATTAG
 AACACAACCT ACCTGTGGTG GGTAAATAAT CAGAGCCTCC CGGTCAGTCC
 10 CAGGCTGCAG CTGTCCAATG ACAACAGGAC CCTCACTCTA CTCAGTGTCA
 CAAGGAATGA TGTAGGACCC TATGAGTGTG GAATCCAGAA CGAATTAAGT
 15 GTTGACCACA GCGACCCAGT CATCCTGAAT GTCCTCTATG GCCCAGACGA
 CCCCACCATT TCCCCCTCAT ACACCTATTAA CCGTCCAGGG GTGAACCTCA
 GCCTCTCCTG CCATGCAGCC TCTAACCCAC CTGCACAGTA TTCTGGCTG
 20 ATTGATGGGA ACATCCAGCA ACACACACAA GAGCTCTTA TCTCCAACAT
 CACTGAGAAG AACAGCGGAC TCTATACCTG CCAGGCCAAT AACTCAGCCA
 25 GTGGCCACAG CAGGACTACA GTCAAGACAA TCACAGTCTC TGCGGAGCTG
 CCCAAGCCCT CCATCTCCAG CAACAACTCC AAACCCGTGG AGGACAAGGA
 30 TGCTGTGGCC TTCACCTGTG AACCTGAGGC TCAGAACACA ACCTACCTGT
 GGTGGGTAAA TGGTCAGAGC CTCCCAGTCA GTCCCAGGCT GCAGCTGTCC
 AATGGCAACA GGACCCTCAC TCTATTCAAT GTCACAAAGAA ATGACGCAAG
 35 AGCCTATGTA TGTGGAATCC AGAACTCAGT GAGTGCAAAC CGCAGTGACC
 CAGTCACCCCT GGATGTCCCTC TATGGGCCGG ACACCCCCAT CATTCCCCC
 40 CCAGACTCGT CTTACCTTTC GGGAGCGAAC CTCAACCTCT CCTGCCACTC
 GGCCTCTAAC CCATCCCCGC AGTATTCTTG GCGTATCAAT GGGATAACCGC
 45 AGCAACACAC ACAAGTTCTC TTTATCGCCA AAATCACGCC AAATAATAAC
 GGGACCTATG CCTGTTTGT CTCTAACTTG GCTACTGGCC GCAATAATTG
 50 55 CATAGTCAAG AGCATCACAG TCTCTGCATAG [SEQ ID NO: 2]
 or a functional equivalent thereof.
 5. A recombinant vector comprising a DNA having a nucleotide sequence encoding a recombinant CEA glycoprotein derivative according to claim 1 or 2, which recombinant vector is capable of directing the expression of the said DNA in a compatible host cell.

6. A transformed host cell containing a recombinant vector comprising a DNA having a nucleotide sequence encoding a recombinant CEA glycoprotein derivative according to claim 1 or 2, which transformed host cell is capable of expressing the said DNA.
- 5 7. A recombinant CEA glycoprotein derivative according to claim 1 or 2 for diagnostic purposes.
8. A reagent for the diagnosis of neoplastic diseases comprising a recombinant CEA glycoprotein derivative as claimed in claim 1 or 2 and an inert carrier material.
- 10 9. A process for the production of a recombinant CEA glycoprotein as claimed in claim 1 or 2, , which process comprises:
 - (a) culturing a host cell containing a recombinant vector comprising a DNA having a nucleotide sequence encoding the said recombinant CEA glycoprotein derivative, such as the DNA having the nucleotide sequence [SEQ ID NO: 2] or an equivalent sequence thereof, under conditions in which the DNA is expressed; and
 - 15 (b) isolating the recombinant CEA glycoprotein derivative produced by the host cell from the culture.
10. A process for the production of a recombinant vector comprising a DNA having a nucleotide sequence encoding a recombinant CEA glycoprotein as claimed in claim 1 or 2, which process comprises:
 - 20 (a) inserting a DNA having a nucleotide sequence encoding the recombinant CEA glycoprotein derivative into a vector;
 - (b) replicating the said vector in a host cell; and
 - (c) isolating the recombinant vector from the host cell.
- 25 11. A method for preparing a reagent for the diagnosis of neoplastic diseases, characterized in that a recombinant CEA glycoprotein derivative as claimed in claim 1 or 2 is mixed with an inert carrier material.
12. A method for diagnosis of a neoplastic disease in a biological sample which method comprises
 - 30 measuring the CEA levels in said sample by using a recombinant CEA glycoprotein derivative according to claim 1 or 2 in an immunoassay.
13. The use of a recombinant CEA glycoprotein derivative as claimed in claim 1 or 2 for determining the presence of tumor cells in a biological sample, e.g. a sample of a body fluid.
- 35 14. A recombinant CEA glycoprotein derivative as claimed in claim 1 or 2, whenever prepared by a process as claimed in claim 9.
15. A recombinant vector comprising a DNA having a nucleotide sequence encoding a recombinant CEA glycoprotein as claimed in claim 1 or 2, whenever prepared by a process as claimed in claim 10.
- 40 16. A test-kit for the diagnosis of neoplastic diseases, which test-kit comprises in a container a recombinant CEA glycoprotein as claimed in claim 1 or 2, if necessary in combination with an inert carrier material, and if necessary additional reagents such as e.g. a monoclonal or a polyclonal antibody directed against CEA.
- 45 17. The invention as hereinbefore described.

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Fig. 1/4

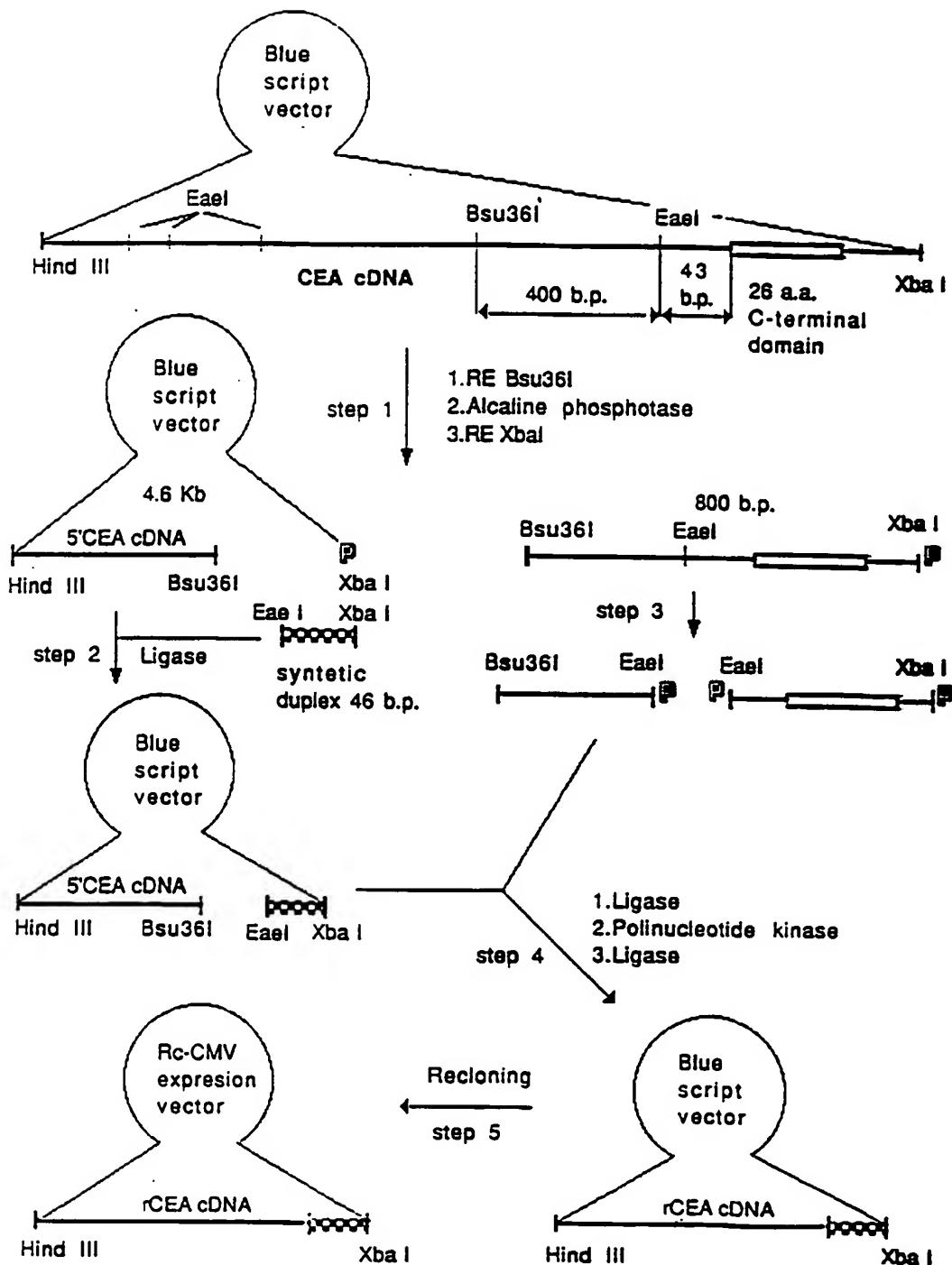


Fig. 2/4

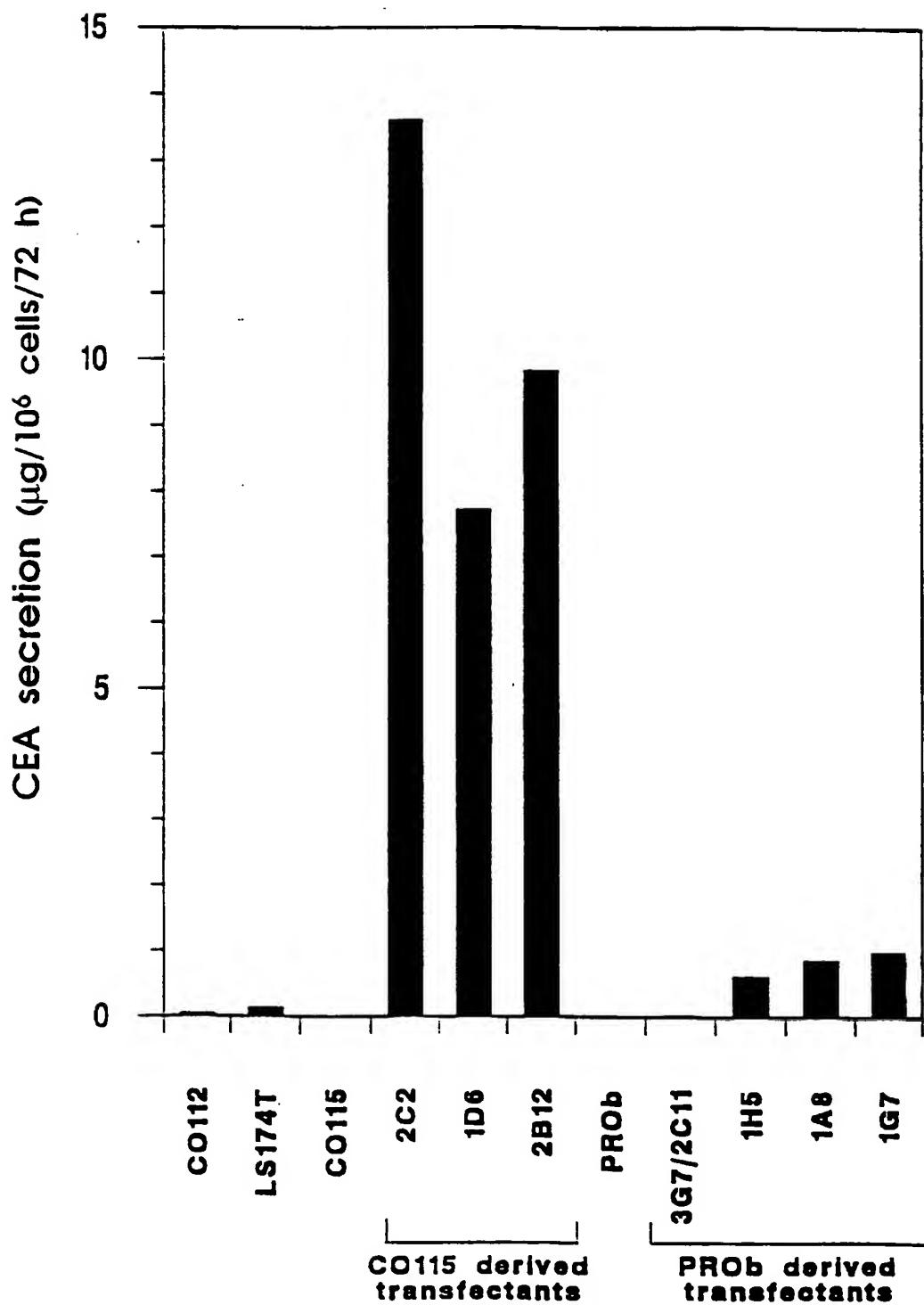


Fig. 3/4

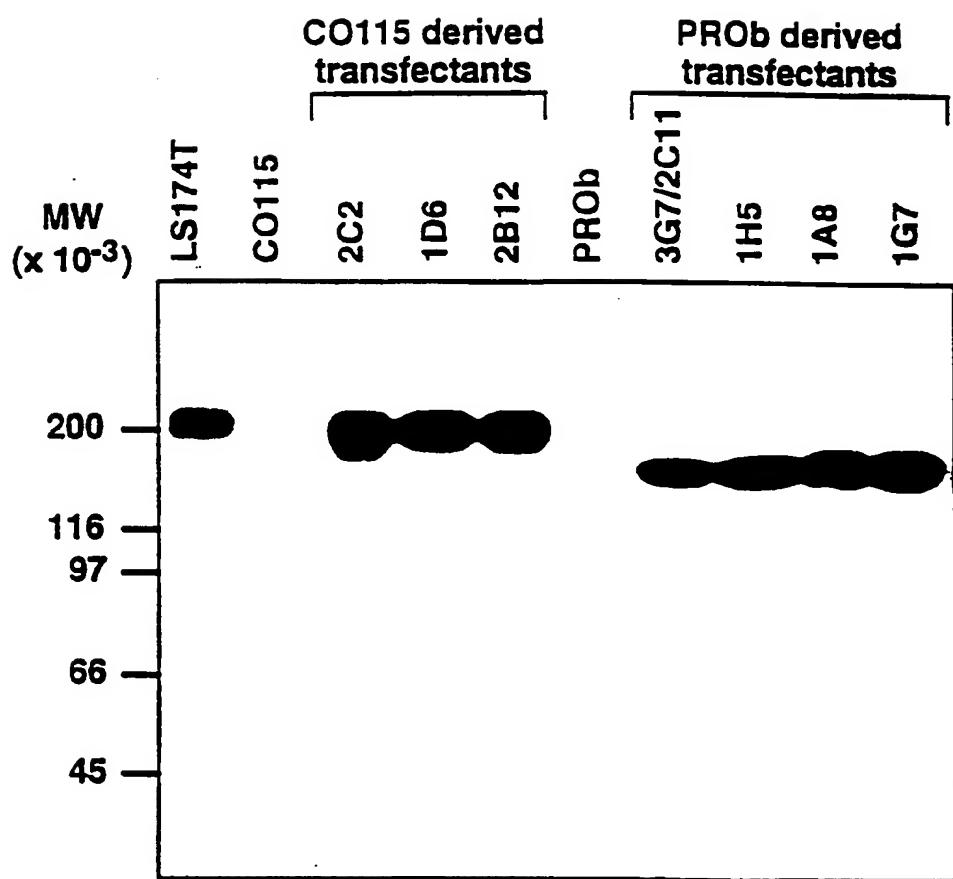
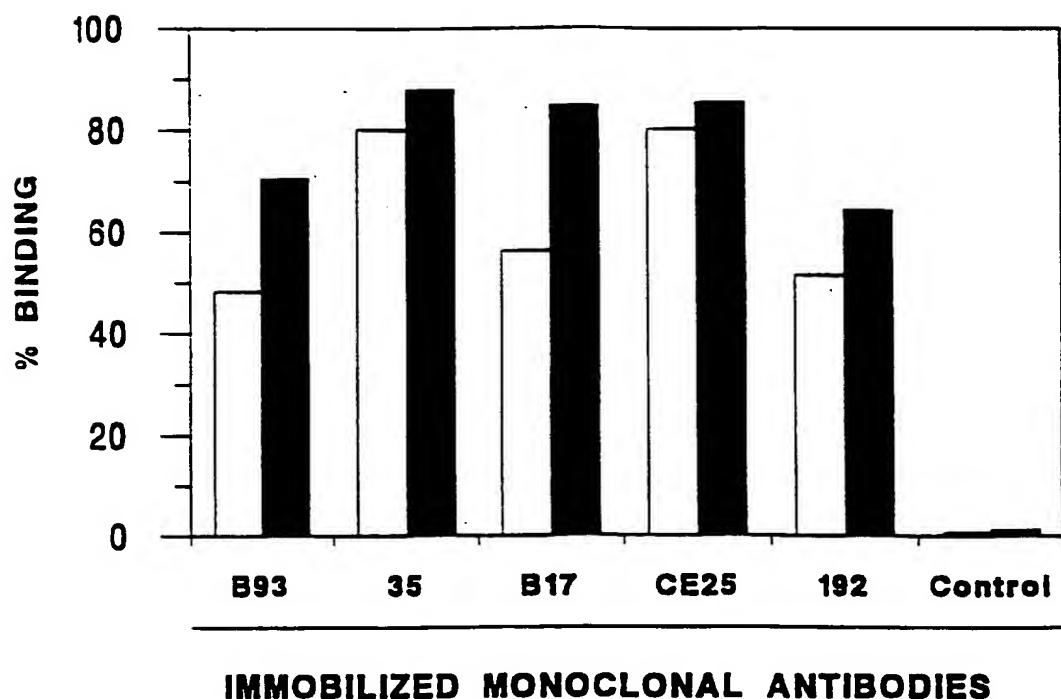


Fig. 4/4





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EUROPEAN SEARCH REPORT

Application Number
EP 94 10 3986

DOCUMENTS CONSIDERED TO BE RELEVANT									
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)						
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 155, no. 2 , 15 September 1988 , DULUTH, MINNESOTA US pages 794 - 800 JEAN F;MALAPERT P;ROUGON G;BARBET J; 'Cell membrane, but not circulating, carcinoembryonic antigen is linked to a phosphatidylinositol-containing hydrophobic domain.' * the whole document * ---	1-17	C12N15/12 C07K13/00 C12N5/10 G01N33/68 C12P21/08						
Y	PATENT ABSTRACTS OF JAPAN vol. 12, no. 450 (C-547)25 November 1988 & JP-A-63 177 794 (SUNTORY LTD.) 21 July 1988 * abstract * ---	1-17							
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, no. 13 , July 1988 , WASHINGTON US pages 4648 - 4652 HEFTA SA;HEFTA LJ;LEE TD;PAXTON RJ;SHIVELY JE; 'Carcinoembryonic antigen is anchored to membranes by covalent attachment to a glycosylphosphatidylinositol moiety: identification of the ethanolamine linkage site.' * the whole document * ---	1-17	TECHNICAL FIELDS SEARCHED (Int.Cl.5) C12N C07K G01N						
Y	EP-A-0 263 933 (MOLECULAR DIAGNOSTICS, INC.; US) 20 April 1988 * the whole document * ----	1-17 -/-							
<p>The present search report has been drawn up for all claims</p> <table border="1"> <tr> <td>Place of search</td> <td>Date of completion of the search</td> <td>Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>29 June 1994</td> <td>Nauche, S</td> </tr> </table>				Place of search	Date of completion of the search	Examiner	THE HAGUE	29 June 1994	Nauche, S
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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.)						
Y	IMMUNOLOGICAL INVESTIGATIONS vol. 21, no. 3 , 1992 pages 241 - 257 KUROKI, M. ET AL.; 'Immunoreactivity of recombinant carcinoembryonic antigen proteins expressed in Escherichia Coli' * the whole document * ---	1-17							
A	FR-A-2 095 728 (F. HOFFMANN LA ROCHE & CIE, CH) 11 February 1972 ---								
P,X	MOLECULAR IMMUNOLOGY vol. 30, no. 10 , July 1993 pages 921 - 927 TERSKIKH A;MACH JP;PELEGREN A; 'Marked increase in the secretion of a fully antigenic recombinant carcinoembryonic antigen obtained by deletion of its hydrophobic tail.' * the whole document *	1-17							
Y	EP-A-0 346 710 (MOLECULAR DIAGNOSTICS, INC;US) 20 December 1989 * the whole document *	1-17	TECHNICAL FIELDS SEARCHED (Int.Cl.)						
<p>The present search report has been drawn up for all claims</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 34%;">Examiner</td> </tr> <tr> <td>THE HAGUE</td> <td>29 June 1994</td> <td>Nauche, S</td> </tr> </table>				Place of search	Date of completion of the search	Examiner	THE HAGUE	29 June 1994	Nauche, S
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